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# The Relationship between Environmental Exposures to Phthalates and DNA Damage in Human Sperm Using the Neutral Comet Assay

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Phthalates are industrial chemicals widely used in many commercial applications. The general population is exposed to phthalates through consumer products as well as through diet and medical treatments. To determine whether environmental levels of phthalates are associated with altered DNA integrity in human sperm, we selected a population without identified sources of exposure to phthalates. One hundred sixty-eight subjects recruited from the Massachusetts General Hospital Andrology Laboratory provided a semen and a urine sample. Eight phthalate metabolites were measured in urine by using high-performance liquid chromatography and tandem mass spectrometry; data were corrected for urine dilution by adjusting for specific gravity. The neutral single-cell microgel electrophoresis assay (comet assay) was used to measure DNA integrity in sperm. VisComet image analysis software was used to measure comet extent, a measure of total comet length (micrometers); percent DNA in tail (tail%), a measure of the proportion of total DNA present in the comet tail; and tail distributed moment (TDM), an integrated measure of length and intensity (micrometers). For an interquartile range increase in specific gravity-adjusted monoethyl phthalate (MEP) level, the comet extent increased significantly by 3.6  $\mu\text{m}$  [95% confidence interval (95% CI), 0.74–6.47]; the TDM also increased 1.2  $\mu\text{m}$  (95% CI, –0.05 to 2.38) but was of borderline significance. Monobutyl, monobenzyl, monomethyl, and mono-2-ethylhexyl phthalates were not significantly associated with comet assay parameters. In conclusion, this study represents the first human data to demonstrate that urinary MEP, at environmental levels, is associated with increased DNA damage in sperm. **Key words:** comet assay, DNA damage, environmental, human sperm, phthalates, urinary metabolites. *Environ Health Perspect* 111:1164–1169 (2003). doi:10.1289/ehp.5756 available via <http://dx.doi.org/> [Online 6 December 2002]

Phthalates are multifunctional chemicals used to hold color and scent in consumer and personal care products (Koo et al. 2002); as carpet backing and as solvents in paints, glue, and insect repellents (ATSDR 1999); and to soften a wide range of plastic goods (Bradbury 1996). Di(2-ethylhexyl) phthalate (DEHP), one of the more commonly used phthalates, leaches from blood products, intravenous and dialysate bags, and tubing made with polyvinyl chloride (Nässberger et al. 1987). Phthalates are also present in drinking water, air, and food (ATSDR 1995, 1999, 2000). Despite the rapid metabolism and elimination of most phthalates (Koo et al. 2002; Nässberger et al. 1987; Peck and Albro 1982), theoretically a constant steady state may be reached because of chronic and repetitive, low-level exposures from dietary ingestion and from many commonly used products.

Evidence of widespread exposure of the U.S. population to phthalates comes from two recent studies on the levels of phthalate metabolites in urine samples collected for the Third National Health and Nutrition Examination Survey (NHANES III) (Blount et al. 2000b) and NHANES 1999 (CDC 2001). The NHANES surveys collect biological samples and information about the health and diet of people in the United States (National Center

for Health Statistics 2001). Four phthalate metabolites—monoethyl phthalate (MEP), mono-2-ethylhexyl phthalate (MEHP), mono-*n*-butyl phthalate (MBP), and monobenzyl phthalate (MBzP)—were present in more than 75% of U.S. subjects sampled (Blount et al. 2000b; CDC 2001).

Evidence of general population exposure to phthalates (Blount et al. 2000b; CDC 2001), as well as *in vitro* studies suggesting that some phthalates are hormonally active (Harris et al. 1997; Nakai et al. 1999) and animal studies showing associations between some phthalates and testicular toxicity (Gangolli 1982; Li et al. 1998; Parks et al. 2000; Sharpe et al. 1995; Thomas et al. 1982), has generated both public and scientific concern about potential reproductive effects of phthalates. Recent *in vitro* studies using the alkaline comet assay (single-cell gel electrophoresis) found di-*n*-butyl phthalate (DBP) and di-isobutyl phthalate (DiBP) to be genotoxic in human epithelial cells of the upper aerodigestive tract (Kleinsasser et al. 2000a), as well as in mucosal cells and lymphocytes (Kleinsasser et al. 2000b). Additionally, the comet assay was used to detect DNA damage in human lymphocytes induced by *in vitro* exposure to DEHP and MEHP (Anderson et al. 1999).

A lack of consensus on which semen quality tests are the best predictors of human male fertility has led to the development of several new methods to evaluate semen quality. The traditional semen analysis measures sperm concentration, motility, and morphology (World Health Organization 1999). Several laboratory techniques are used to evaluate sperm DNA, such as the sperm chromatin structure assay (SCSA) (Evenson et al. 1991). The SCSA may prove to be a useful clinical test because of its high repeatability and its ability to measure an aspect of fertility that differs from what can be offered by the traditional semen analysis (Evenson et al. 1999). Other DNA tests include fluorescence *in situ* hybridization, used to measure aneuploidy, as well as assays used to measure DNA integrity, including single-cell microgel electrophoresis (comet assay) and the terminal deoxynucleotidyl transferase-mediated dUTP-biotin end-labeling (TUNEL) assay (Lähdetie et al. 1996; Martin 1993; Sun et al. 1997; World Health Organization 1999).

Few published human studies have examined the effect of environmental chemicals on DNA integrity in sperm as measured by the comet assay. In the present study, we used the neutral comet assay to measure DNA integrity in human sperm and investigated whether DNA integrity was associated with urinary concentrations of five phthalate monoesters.

## Materials and Methods

**Subjects.** The study was approved by the Harvard School of Public Health and Massachusetts General Hospital (MGH) Human Subjects Committee, and all subjects signed an informed consent form. Subjects were recruited from an ongoing semen quality

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study and are male partners of a subfertile couple who presented to the MGH Andrology Laboratory (Boston, MA) between January 2000 and October 2001 for semen analysis as part of an infertility investigation. Eligible men were those between 20 and 54 years old. Men presenting for postvasectomy semen analysis were excluded.

**Semen sample collection.** Semen was produced on site at MGH by masturbation into a sterile plastic specimen cup after a recommended period of abstinence of 48 hr. After liquefaction at 37°C for 30 min, pH, color, and viscosity measurements were made, and semen was analyzed. Sperm concentration and motility were measured by computer-aided sperm analysis (version 10HTM-IVOS; Hamilton-Thorn, Beverly, MA) using manufacturer instructions, and morphology was measured manually using Kruger strict criteria. Remaining raw semen was then frozen in 0.25-mL cryogenic straws (CryoBiosystem, I.M.V. Division, San Diego, CA) by immersing the straws directly into liquid nitrogen (−196°C). Previous work in our laboratory showed that this freezing method produced results that were highly correlated with results from fresh, unfrozen samples (Duty et al. 2002). The straws were thawed by gently shaking in a 37°C water bath for 10 sec, and the semen was immediately processed for comet assay.

**Comet assay.** The entire procedure was conducted under low indirect incandescent light (60 W) to minimize light-induced damage to sperm DNA. All chemicals were purchased from VWR Scientific (West Chester, PA) unless otherwise specified. After thawing, semen (with approximately  $2 \times 10^5$  sperm) was mixed with 400  $\mu$ L 0.7% agarose (3:1 high resolution; Amresco, Solon, OH). Fifty microliters of this semen/agarose mixture was embedded between two additional 200- $\mu$ L layers of 0.7% agarose on specially designed, partially frosted, microgel electrophoresis glass slides with a clear central window (Erie Scientific, Portsmouth, NH). Cover glasses were removed before submersion of slides in a cold lysing solution (4°C) of 2.5 M NaCl, 100 mM EDTA tetrasodium salt, 10 mM Tris-base (pH 10), 1% sodium lauroyl sarcosine, and 1% Triton X-100 (Roche Diagnostics Corp., Indianapolis, IN); this step mainly dissolves the cell membrane to make chromatin accessible for the next two enzyme digestion steps. The slides were then transferred to enzyme treatment (2.5 M NaCl, 5 mM Tris, 0.05% sodium lauroyl sarcosine with pH adjusted to 7.4), and 10 mg/mL of RNase (Amresco, Solon, OH). After 4 hr at 37°C, the slides were transferred into enzyme treatment plus 1 mg/mL DNase-free proteinase K (Amresco, Solon, OH) for 18 hr at 37°C. These two steps are crucial for decondensing sperm chromatin and allowing migration of

broken DNA out of the nucleus. Slides were then equilibrated in neutral electrophoresis solution (300 mM sodium acetate, 100 mM Tris, pH 9) for 20 min before being electrophoresed under neutral conditions at 12 V and 130 mA for 1 hr at room temperature. This was followed by precipitation and fixation of cells first in absolute alcohol mixed with 10 M ammonium acetate for 15 min, and then in 70% ethanol with 100 mg of spermine for 30 min. The resulting slides were air dried and then stained with YOYO dye (Molecular Probes, Eugene, OR), an intensely fluorescent DNA dye. Fluorescent comet patterns were examined with a Leica fluorescence microscope model DMLB under 400 $\times$  magnification and fluoroisothiocyanate filter combination.

**Image analysis.** VisComet image analysis software, kindly donated by Impuls Computergestützte Bildanalyse GmbH (Gilching, Germany) was used to measure comet extent, percent DNA in tail (tail%), and tail distributed moment (TDM) on 100 sperm in each semen sample. Comet extent is a measure of total comet length from the beginning of the head to the last visible pixel in the tail. This measurement is similar to that obtained by manual analysis using an eyepiece micrometer. Tail% is a measurement of the proportion of the total DNA that is present in the tail. The TDM is an integrated value that takes into account both the distance and intensity of comet fragments. The formula used to calculate the TDM is

$$M_{\text{dist}} = \Sigma (I \cdot X) / \Sigma I,$$

where  $\Sigma I$  is the sum of all intensity values that belong to the head, body, or tail, and  $X$  is the  $x$ -position of intensity value. In addition to these two parameters, cells too long to measure with VisComet ( $> 300 \mu\text{m}$ ; “long cells”) were tallied and used as a third measure of DNA damage. Because of the presence of long cells in most subjects, more than 100 cells may have been screened and scored to allow for the measurement of comet extent, tail%, and TDM on 100 cells per subject.

**Urinary phthalate metabolites.** We measured the monoester phthalate metabolites because of potential sample contamination from the ubiquitous parent diester and because the metabolites are believed to be the active toxicant, not the parent diester compounds (Li et al. 1998; Peck and Albro 1982). Eight urinary phthalate metabolites—MEP, monomethyl phthalate (MMP), MEHP, MBP, MBzP, mono- $n$ -octyl phthalate (MOP), mono-3-methyl-5-dimethylhexyl (isononyl) phthalate (MINP), and monocyclohexyl phthalate (MCHP)—were measured in a single spot urine sample, collected in a sterile specimen cup on the same day as the semen sample. Because more than 75% of the study

population had levels of MCHP, MINP, and MOP below the limit of detection (LOD), the results for these metabolites were not informative and are not included in the analysis. The analytical approach has been described in detail elsewhere (Blount et al. 2000a). Briefly, urinary phthalate metabolite determination involved enzymatic deconjugation of metabolites from the glucuronidated form, solid-phase extraction, separation with high-performance liquid chromatography, and detection by tandem mass spectrometry. Detection limits were in the low nanogram per milliliter range. Reagent blanks and  $^{13}\text{C}_4$ -labeled internal standards were used along with conjugated internal standards to increase precision of measurements. One method blank, two quality control samples (human urine spiked with phthalates), and two standards were analyzed along with every 10 unknown urine samples (Blount et al. 2000a). Analysts were blind to all information concerning subjects.

**Specific gravity adjustment.** We measured urinary specific gravity to identify unreliable urine samples and to normalize phthalate levels for differences in urinary dilution between subjects. We used a hand-held refractometer (National Instrument Company, Inc., Baltimore, MD) that was calibrated with deionized water before each batch of measurements. Phthalate concentrations were corrected for specific gravity by the formula

$$P_c = P[(1.024 - 1)/(SG - 1)],$$

where  $P_c$  is the specific gravity-corrected phthalate concentration (nanograms per milliliter),  $P$  is the observed phthalate concentration (nanograms per milliliter), and  $SG$  is the specific gravity of sample (Boeniger et al. 1993; Teass et al. 1998). Specific gravity-adjusted phthalate levels were used in statistical modeling as a continuous predictor variable without transformation.

**Statistical analysis.** For data analysis, we used Statistical Analysis Software (SAS), version 8.1 (SAS Institute Inc., Cary, NC), and we performed descriptive analyses of subject characteristics. In separate univariate and multiple regression analyses, the mean of 100 cells per person was used for each of the dependent variables: comet extent, tail%, and TDM. Because mean comet extent and TDM were normally distributed (Shapiro-Wilk test  $p$ -values  $> 0.35$ ), they were used untransformed in the regression analyses. However, because tail% was not normally distributed, analyses using both untransformed and log-transformed tail% were performed. Because the results and their interpretation did not differ, we chose to present only the untransformed tail% results for ease of interpretation. We used regression analysis to explore the relationship between the comet parameters and specific gravity-adjusted

urinary phthalate metabolite levels, adjusting for covariates. Covariates for inclusion were based on statistical and biologic considerations (Hosmer and Lemeshow 1989). Because the number of long cells in a semen sample was not normally distributed, it was transformed using the arcsine transformation (Zar 1984) and regressed on urinary phthalates. Spearman correlation coefficients were used to determine correlations among phthalate monoesters and among comet parameters.

In the regression models, age was modeled as a continuous independent variable after checking for appropriateness using a quadratic term. Abstinence time was modeled as an ordinal five-category variable (2 or fewer days, 3, 4, 5, and 6 or more days), and smoking status was used as a dummy variable (current and former vs. never). Race was categorized into four groups: white, African American, Hispanic, and other.

## Results

Of the 253 men recruited into an ongoing semen quality study, 1 dropped out and 168 subjects had both phthalate levels and comet analysis results (Figure 1). Because the study initially did not archive semen for future comet assay analyses, the first 46 subjects recruited were excluded even though their urine had been collected and analyzed for phthalates. An additional 17 subjects were excluded from the data analysis because they could not provide a urine sample at the time of semen collection, and 12 subjects with archived semen samples had no sperm (azoospermic), and so the comet assay

could not be performed. Nine samples were lost when cryostraws exploded upon thawing.

Demographic information and semen parameters are given in Table 1. The mean ( $\pm$  SD) of age and body mass index of the 168 subjects was  $36.3 \pm 5.7$  years and  $28.2 \pm 4.6$  years, respectively. About 77% of subjects were white, 7.8% African American, 7.2% Hispanic, and 7.8% other. Most subjects (72.0%) never smoked, and only 9.5% were current smokers (smoked within the past month). The mean ( $\pm$  SD) semen concentration, motility, and strict morphology were  $111.1 \pm 91.0$  million/mL,  $52.4 \pm 23.6\%$  motile sperm, and  $7.1 \pm 4.5\%$  normally shaped sperm, respectively. Although the mean values are all larger than the reference values for each semen parameter [World Health Organization

(WHO) 1999], 52% of subjects had values for one or more semen parameters below the WHO reference values. Twenty-four subjects (14.3%) had  $< 20$  million sperm/mL, 68 subjects (40.5%) had  $< 50\%$  motile sperm, and 38 subjects (22.6%) had  $< 4\%$  normally shaped sperm. Eighty-one subjects (48%) had semen parameters that were above the WHO reference values for all three semen parameters.

The distribution of comet parameters and specific gravity-adjusted urinary phthalate metabolite levels are shown in Table 2. Of the 168 subjects with both comet assay results and urinary phthalate monoester levels, 27 were excluded from the primary data analysis because specific gravity values were outside the acceptable range ( $< 1.010$  or  $> 1.030$ ) (Boeniger et al. 1993; Teass et al. 1998). MEP was detected in

**Table 1.** Subject demographics and semen and comet parameters ( $n = 168$ ).

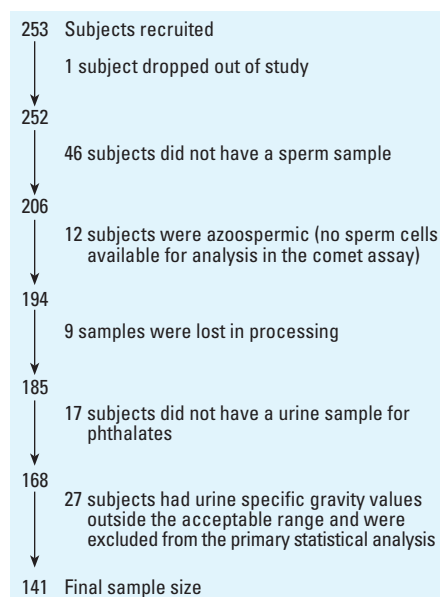
Parameters	Mean $\pm$ SD	No. (%)
Age	$36.3 \pm 5.7$	
Body mass index	$28.2 \pm 4.6$	
Race <sup>a</sup>		
White		129 (77.2)
African American		13 (7.8)
Hispanic		12 (7.2)
Other		13 (7.8)
Smoking status		
Never smoker		121 (72.0)
Ever smoker		47 (28.0)
Current smoker		16 (9.5)
Ex-smoker		31 (18.5)
Semen parameters		
Sperm concentration (million/mL)	$111.1 \pm 91.0$	
Subjects $< 20$ million sperm/mL		24 (14.3)
Sperm motility (% motile)	$52.4 \pm 23.6$	
Subjects $< 50\%$ motile sperm		68 (40.5)
Sperm morphology <sup>b</sup> (% normal morphology)	$7.1 \pm 4.5$	
Subjects $< 4\%$ normal morphology		38 (22.6)
Comet assay parameters <sup>c</sup>		
Comet extent ( $\mu$ m)	$125.3 \pm 32.3$	
Tail%	$20.9 \pm 7.7$	
TDM ( $\mu$ m)	$59.0 \pm 13.7$	
Number of long cells	$10.0 \pm 12.0$	
Subjects without long cells		17 (10.1)
Subjects with long cells		151 (89.9)

<sup>a</sup>One person missing race. <sup>b</sup>Kruger strict criteria used for morphology determination. <sup>c</sup>VisComet image analysis software was used to measure comet extent (microns), tail%, and TDM. One person missing both TDM and tail%.

**Table 2.** Distribution of urinary phthalate levels<sup>a</sup> and comet assay parameters<sup>b</sup>.

	No. <sup>c</sup>	Minimum	Percentile					Maximum	Geometric mean
Phthalate monoester <sup>d</sup>			5th	25th	50th	75th	95th		
MEP	141	9.8	27.6	59.6	160.5	503	2029.8	5396.2	186.8
MBzP	141	$< \text{LOD}$	1.5	3.9	8.9	15.6	39.8	138.3	7.8
MBP	141	$< \text{LOD}$	3.9	10.7	18.1	31.0	69.9	2623.4	18.2
MEHP	141	$< \text{LOD}$	$< \text{LOD}$	1.9	5.2	13.8	86.0	319.3	7.1
MMP	141	$< \text{LOD}$	$< \text{LOD}$	2.1	4.9	11.4	32.1	104.7	6.1
Comet parameters									
Comet extent	141	53.4	72.8	105.7	124.8	149.8	180.0	219.2	121.5
Tail%	140	9.9	12.8	16.6	19.8	22.5	35.1	61.6	19.9
TDM	140	29.5	37.4	48.8	58.8	68.7	82.6	91.2	57.4
Number of long cells	141	0	0	2	6	13	31	73	6.8

<sup>a</sup>Specific gravity-adjusted urinary phthalate monoester concentration (ng/mL urine). <sup>b</sup>VisComet image analysis software was used to measure comet extent, tail%, and TDM. <sup>c</sup>Twenty-seven of 168 subjects were excluded because specific gravity value was out of acceptable range ( $< 1.010$  or  $> 1.030$ ), and one subject was missing TDM and tail% measures. <sup>d</sup>LODs for phthalates (ng/mL) are as follows: MEP, 1.0; MBzP, 0.8; MBP, 0.6; MEHP, 1.2; MMP, 0.71.



**Figure 1.** Subject exclusions. Of the 253 subjects recruited to participate, 168 subjects had semen and urine samples available for analysis. The final sample size for statistical analysis was 141 subjects; 27 subjects were excluded because urine specific gravity was out of the acceptable range ( $< 1.010$  or  $> 1.030$ ).



100% of subjects, MBP and MBzP in at least 95% of subjects, and MEHP and MMP in at least 75% of subjects. The phthalate monoester with the highest concentration was MEP, ranging from 9.8 to 5396.2 (ng/mL) ppb with a geometric mean of 186.8 ppb. The median MEP concentration ranged from 9- to 32-fold higher than any other phthalate metabolite. Interquartile ranges (IQRs) varied considerably among the phthalates, from 443 ppb for MEP to only 9.3 ppb for MMP. The IQR of comet parameters also varied, 44  $\mu\text{m}$  for comet extent and 20  $\mu\text{m}$  for TDM. Fifty percent of comet extents were between 105 and 150  $\mu\text{m}$ , with < 5% of cells longer than 180  $\mu\text{m}$  or shorter than 70  $\mu\text{m}$ . Figure 2A–C demonstrates the heterogeneity of comet tail lengths within an individual; Figure 2D depicts the comet cell referred to as a “long cell,” a cell that was too long to measure with image analysis software.

The mean ( $\pm$  SD) comet extent, tail%, and TDM were  $125.3 \pm 32.3$   $\mu\text{m}$ ,  $20.9 \pm 7.7\%$ , and  $59.0 \pm 13.7$   $\mu\text{m}$ , respectively. Comet extent ranged from 53.4 to 219.2  $\mu\text{m}$ , tail% from 9.9 to 61.6%, and TDM from 29.5 to 91.2  $\mu\text{m}$ . The number of long cells in a semen sample ranged from 0 to 73 cells. We counted the number of long cells in addition to the 100 cells measured with the VisComet software per sample. Comet extent and TDM were highly correlated ( $r = 0.90$ ,  $p < 0.0001$ ); however, tail% was moderately correlated with comet extent ( $r = 0.35$ ,  $p < 0.0001$ ) and weakly correlated with TDM ( $r = 0.14$ ;  $p = 0.10$ ). Moderate correlations existed between the number of long cells and both comet extent and TDM ( $r = 0.45$  and  $r = 0.44$ , respectively;  $p < 0.0001$ ), but the correlation between long cells and tail% was weak ( $r = 0.10$ ,  $p = 0.26$ ). The five phthalate monoesters were only weakly or moderately correlated with each other. The strongest correlation was found between MBP and MBzP ( $r = 0.43$ ;  $p < 0.001$ ), which is expected because the diester butyl benzyl phthalate (BBzP) gives rise to both MBP and MBzP in a 5:3 ratio (NTP 2000). The weakest correlation was found between MMP and MBzP ( $r = 0.015$ ,  $p = 0.9$ ), suggesting that exposures to these phthalates may come from different sources.

In the univariate linear regression analyses, although not statistically significant, comet extent and TDM were longer in current smokers than in never smokers (127.7  $\mu\text{m}$  vs. 125.5  $\mu\text{m}$ , and 63.0  $\mu\text{m}$  vs. 59.3  $\mu\text{m}$ , respectively). Tail% was higher in former smokers but lower in current smokers compared with never smokers (23.5% and 18.4% vs. 20.1%, respectively;  $p = 0.03$  and 0.44, respectively). The relationships between age and both comet extent and TDM were inconsistent and not significant. Comet extent increased 0.007  $\mu\text{m}/\text{year}$  [95% confidence interval (CI),  $-0.92$  to 1.06], but TDM decreased 0.14  $\mu\text{m}/\text{year}$  (95% CI,  $-0.56$  to 0.27). Tail% significantly

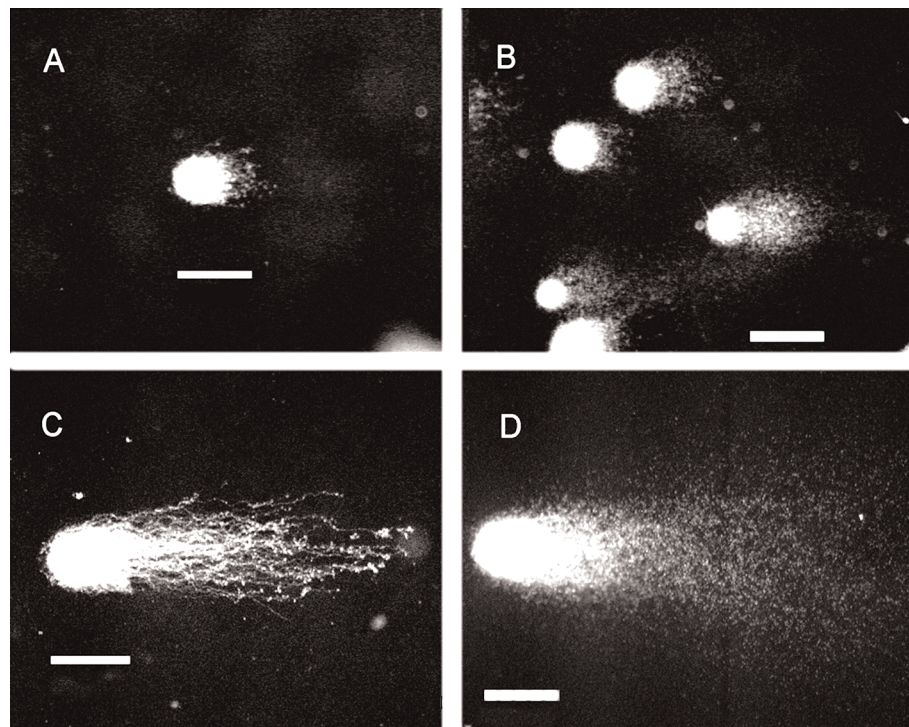
increased 0.22%/year (95% CI, 0.00 to 0.44). The number of long cells increased marginally as age increased ( $< 1$  cell/year,  $p = 0.07$ ), but it was not associated with smoking. In contrast to the unstable relationships between age and smoking with comet assay parameters, MEP was significantly associated with comet extent; the regression coefficient was 3.5  $\mu\text{m}/\text{IQR}$  (95% CI, 0.73 to 6.33). Tail% was not significantly associated with MEP ( $-0.11\%/ \text{IQR}$ ; 95% CI,  $-0.78$  to 0.56). The relationship between TDM and MEP and MBzP was less stable and failed to reach statistical significance, with increases of 1.10  $\mu\text{m}/\text{IQR}$  (95% CI,  $-0.10$  to 2.30) and 1.18  $\mu\text{m}/\text{IQR}$  (95% CI,  $-0.25$  to 2.62), respectively. There were no significant, or even suggestive, univariate relationships between specific gravity-adjusted phthalate levels and the number of long cells. The regression coefficients were close to zero, and the confidence intervals were wide.

Although the relationships between smoking and comet assay parameters were inconsistent, we included smoking as a potential confounder in the multiple regression models because several studies have reported increased DNA damage in smokers (Fraga et al. 1996; Sun et al. 1997; Ünder et al. 1999). Additionally, age was included in the multiple regression models because there is evidence that DNA damage increases with age (Møller et al. 2000; Singh et al. 2001). Generally, the crude and adjusted coefficients in the multiple regression models were similar, indicating that

there was minimal confounding by age and smoking status.

The final multiple regression models are summarized in Table 3. After adjusting for age and smoking status, for an IQR increase in specific gravity-adjusted MEP concentrations, the comet extent significantly increased 3.61  $\mu\text{m}$  (95% CI, 0.74 to 6.47), whereas TDM increased 1.17  $\mu\text{m}$  but was of borderline statistical significance (95% CI,  $-0.05$  to 2.38). Tail% decreased marginally with an IQR change in MEP, although it was not significant ( $-0.17\%/ \text{IQR}$ ; 95% CI,  $-0.81$  to 0.47). In contrast, the coefficients for the relationships between MBP and MEHP and comet extent, tail%, and TDM were near zero and not significant. In addition, the coefficients for the adjusted relationships between phthalate levels and the number of long cells were close to zero and nonsignificant (data not shown).

In a sensitivity analysis, we reanalyzed the data after including the 27 subjects that were excluded from the primary analysis because their urine specific gravity was outside the acceptable range. In the reanalysis, the coefficients for the relationships between MEP and comet extent and TDM, adjusted for age and smoking, were statistically significant and became larger, 3.67  $\mu\text{m}/\text{IQR}$  (95% CI, 1.07 to 6.26) and 1.23  $\mu\text{m}/\text{IQR}$  (95% CI, 0.12 to 2.34), respectively. The relationship between MEP and tail% remained essentially unchanged ( $-0.09\%/ \text{IQR}$ ; 95% CI,  $-0.66$  to 0.48). The



**Figure 2.** Comet tail length. (A) Cell with a short comet tail. (B) Considerable heterogeneity of comet tail lengths within an individual. (C) Cell with a long comet tail. (D) Comet referred to as a “long cell,” a cell with highly fragmented DNA that was too long to measure with image analysis software. Bars = 50  $\mu\text{m}$ .

regression coefficients for the relationship between MBzP and comet extent and TDM increased moderately in magnitude, to 2.89  $\mu\text{m}/\text{IQR}$  (95% CI, -0.10 to 5.87) for comet extent and to 1.20  $\mu\text{m}/\text{IQR}$  (95% CI -0.07 to 2.46) for TDM. The coefficients and confidence intervals for MBP and MEHP were similar to the results of the initial analysis, and their interpretation remained unchanged. The coefficients for MMP and comet extent and TDM became smaller in magnitude, although the confidence intervals narrowed.

## Discussion

The present study represents one of the first human studies to report an association between urinary levels of MEP, at levels found in the general population, and increased DNA migration in sperm, assessed using the neutral comet assay. Specifically, there was a statistically significant positive association between urinary MEP and mean comet extent and a suggestive association with TDM. However, no significant associations were found between comet assay parameters and other urinary phthalate metabolites, including MBP, MBzP, MEHP, and MMP.

Animal data suggest that several phthalates, including butyl benzyl phthalate (BBzP), DBP, DEHP, and MEHP, are associated with damage to the testes and decreased sperm production (Gangolli 1982; Li et al. 1998; Parks et al. 2000; Sharpe et al. 1995; Thomas et al. 1982); however, there are only a few studies on the genotoxicity of these agents. Using the alkaline comet assay, researchers have found evidence of genotoxicity with *in vitro* studies examining lymphocytes and mucosal cells of the upper aerodigestive tract after exposure to DBP and DiBP (Kleinsasser et al. 2000a, 2000b, 2001). In another study using the alkaline comet assay on human leukocytes, an association between MEHP and DEHP and increased tail moments was found (Anderson et al. 1999). In contrast to those studies, in the present study we found no linear association between MEHP or MBP and sperm DNA migration. It is unclear whether the different results derive from the different cell types studied or the use of the neutral assay in the present

study, compared with the use of the alkaline assay in the other studies.

In the neutral comet assay, a cell with fragmented DNA has the appearance of a "comet" with a brightly fluorescent head and a fluorescent tail whose intensity represents the relative amount of DNA strand breaks present (Hughes et al. 1997; Singh and Stephens 1998; Singh et al. 1988). The comet assay for human sperm was adapted from methods used on somatic cells, which can be conducted under alkaline or neutral conditions. Neutral conditions were used for human sperm because of the abundance of alkali-sensitive sites in sperm. Alkaline test conditions can induce damage at alkali-labile sites and produce DNA strand breaks (Singh et al. 1989).

In previous studies using the comet assay, changes in DNA migration (comet length) were detected at low levels of x-irradiation, 12.5 centigrays (rads) in human lymphocytes (Singh and Stephens 1997) and 50 centigrays (rads) in human sperm (Duty et al. 2002). Therefore, we considered comet extent and TDM to represent sensitive quantitative measures of DNA damage. However, tail moment is purported to be a more sensitive measure of DNA damage than TDM and comet extent. This increased sensitivity results from observations that with increasing levels of DNA damage, the tail length may not but tail% may continue to increase (McKelvey-Martin et al. 1993). In addition to these traditional comet assay parameters, we also tallied the number of long cells. We hypothesize that the long cell parameter represents an independent measure of DNA damage. This was partially confirmed by the weak correlation with the traditional comet assay parameters. Long cells represent very highly damaged cells. Definitive characterizations of the comet assay parameters and the significance of the long cells remain to be resolved. Although the present study was not designed to investigate this, we felt it was important to quantify long cells as a separate measure because this may prove useful in future studies using the neutral comet assay.

Although the data in the present study suggest an association between MEP and increased DNA migration in the comet assay, they must be interpreted cautiously because the phthalate

levels are based on a single urine sample from a limited number of subjects. A recent study documents good reproducibility of urinary phthalate monoester measurements from day to day (Pearson correlation coefficients ranged from 0.5 to 0.8); however, this was in a small number of subjects ( $n = 46$ ), all of whom were women and African American (Hoppin et al. 2002). Because phthalates have short half-lives (Nässberger et al. 1987; Peck and Albro 1982), spot urine samples reflect recent exposure. However, if a steady state of exposure and biologic burden is achieved with chronic repeated exposures to phthalates through the diet and the use of household and personal care products, then the utility of a single specimen is improved.

Urinary phthalate levels were normalized for urine dilution differences by adjusting for specific gravity. There are several methods to adjust for urine volume (Boeniger et al. 1993; Teass et al. 1998), and although creatinine is a frequently used form of adjustment, it is not always appropriate. If a compound is excreted primarily by tubular secretion, it is not appropriate to adjust for creatinine level (Teass et al. 1998). Although the methods of excretion of the phthalate monoesters measured in this study are unknown, terephthalic acid, a dicarboxylic acid phthalate analog, was found to be actively secreted by renal tubules and actively reabsorbed by the kidney (Tremaine and Quebbemann 1985). Furthermore, because organic compounds that are conjugated with glucuronides in the liver, such as phthalates, are eliminated by active tubular secretion (Boeniger et al. 1993), creatinine adjustment may not be appropriate. Additionally, creatinine levels may be confounded by muscularity, physical activity, urine flow, time of day, diet, and disease states (Boeniger et al. 1993; Teass et al. 1998). For these reasons, specific gravity was used to normalize phthalate levels. We excluded samples with specific gravity less than 1.010 or greater than 1.030 (Teass et al. 1998).

Phthalate levels in the present study were compared with levels measured in NHANES III (Blount et al. 2000b) and NHANES 1999 (CDC 2001). Even after limiting the NHANES III data to only men (Barr D. Personal communication; unpublished data), the phthalate levels were on average two to three times higher than those in the present study. The NHANES 1999 phthalate metabolite levels were also twice as high as those in our study. The two exceptions were MEP, which was similar between studies, and MEHP, which was twice as high in our study. MMP was not measured in NHANES data. It is unclear why MEHP levels were high in the present study because few subjects reported recent medical interventions including intravenous infusions, transfusions, or hemodialysis, which might account for higher MEHP levels. Despite the fact that the levels of phthalate

**Table 3.** Adjusted regression coefficients for a change in comet assay parameters associated with an IQR increase in phthalate monoester levels<sup>a</sup> ( $n^b = 141$ ).

Phthalate monoester	IQR (ng/mL)	Coefficients <sup>c</sup> for comet assay parameters <sup>d</sup>		
		Comet extent (95% CI)	Tail% (95% CI)	TDM (95% CI)
MEP	443	3.61 (0.74 to 6.47)*	-0.17 (-0.81 to 0.47)	1.17 (-0.05 to 2.38)
MBzP	11.7	2.45 (-1.07 to 5.97)	0.05 (-0.07 to 0.82)	1.06 (-0.42 to 2.54)
MBP	20.3	-0.31 (-0.80 to 0.19)	-0.02 (-0.13 to 0.09)	-0.12 (-0.32 to 0.09)
MEHP	11.9	-0.19 (-1.54 to 1.16)	-0.01 (-0.30 to 0.29)	0.08 (-0.48 to 0.65)
MMP	9.3	2.20 (-1.51 to 5.90)	-0.12 (-1.24 to 0.40)	0.93 (-0.62 to 2.49)

<sup>a</sup>Specific gravity-adjusted urinary phthalate monoester concentration (ng/mL urine). <sup>b</sup>27 of 168 subjects were excluded because specific gravity value was out of acceptable range (< 1.010 or > 1.030). <sup>c</sup>Adjusted for age (continuous), and smoking (current, former versus never). <sup>d</sup>VisComet image analysis software was used to measure comet parameters. Coefficient units are (parameter)/IQR. \* $p = 0.015$ .



monoesters differed between our study and both NHANES studies, the metabolites with the highest levels were similar across studies (Blount et al. 2000b; CDC 2001). In all three studies, the highest phthalate levels were for MEP, followed by MBP and then MBzP.

Although the men in the present study may not be representative of men in Massachusetts, generalizability may not necessarily be limited. It is a misconception that generalization from a study group depends on the study group's being a representative subgroup of the target population (Rothman and Greenland 1998). For generalizability to be limited, the relationship between comet parameters and phthalates in this clinic population would need to differ from the relationship in the population being generalized to. We would need to speculate that men in this andrology clinic differ by some factor that alters their response to phthalates. Currently, there is no reason to suspect that men who visit this andrology clinic are more or less "sensitive" to phthalates than men who visit other clinics or men from the general population. However, until the results of the present study are replicated in larger and different populations, the generalizability of our results will remain unclear.

In summary, although a significant association was seen between MEP and one measure of DNA integrity in sperm, these results need to be duplicated in a larger study. The lack of significant associations between comet assay parameters and the other four phthalate metabolites may indicate a true difference in genotoxicity between monoesters. It may also reflect markedly different exposure distributions of these monoesters when compared with the broad exposure distribution of MEP. Conversely, the comet assay associations found with MEP may reflect conducting multiple comparisons. In conclusion, this is the first epidemiologic study to explore the association between urinary monoester phthalates at general population levels and DNA integrity in sperm. In addition, the present study demonstrates that the neutral comet assay is a potentially useful tool for detecting DNA damage in human sperm in epidemiologic studies.

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